INTRODUCTION

Vitamin D metabolism

Vitamin D is synthesized endogenously in the skin as vitamin D_3 (cholecalciferol) when exposed to ultraviolet B (UVB) radiation in sunlight, but small amounts can also be obtained from animal foods such as oily fish, egg and meat [1]. Negligible amounts of vitamin D are obtained as vitamin D_2 (ergocalciferol) from plant sources [2,3]. In the Nordic countries a dietary vitamin D intake of 10 µg/day is recommended to children, adults and pregnant women, but dietary surveys in the Danish population show that those recommendations are not met. The median intake in children is estimated to be less than 2 µg/day, and in adults 2.5 µg/day [4]. Thus, in Northern countries the synthesis of vitamin D in the skin through the summer seems essential to secure a sufficient blood concentration of vitamin D through the winter.

The first step in the vitamin D synthesis in humans is when the UVB radiation (λ = 290-315) reaches the human skin and stimulates the photo conversion of 7-dehydrocholesterol located in the skin to previtamin D_3, which immediately begins to equilibrate thermally into vitamin D_3. Vitamin D_2 and D_3 from diet are together with synthesized D_3 from the skin taken up into the blood stream where it binds to vitamin D binding proteins and lipoproteins for transport to the liver. In the liver it is further 25-hydroxylated by the vitamin D-25-hydroxylase enzyme (CYP2R1) to produce the major circulating prohormone 25-hydroxyvitamin D (25(OH)D) that undergoes further hydroxylation mainly in the kidneys by the 25(OH)D-1α-hydroxylase enzyme (CYP27B1) to form the secosteroid hormone 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) [2,5,6]. 25(OH)D and $1,25(OH)_2D$ may also be catabolized to the inactive metabolites by 24-hydroxylase (CYP24A1) to $24,25(OH)_2D$ and $1,24,25(OH)_3D$, respectively, which are then excreted. The active form of vitamin D, $1,25(OH)_2D$, performs many of its biologic functions in multiple tissues, including effects on hormone secretion, modulation of immune responses, and control of cellular proliferation and differentiation, by regulating gene transcription through the vitamin D receptor (VDR) [7]. The circulating levels of $1,25(OH)_2D$ are tightly regulated by serum calcium, phosphorus, and parathyroid hormone (PTH), which makes the hormone an inadequate indicator of vitamin D status. Instead the status is best reflected by the serum 25(OH)D concentration, which represents the combined amounts of synthesized and dietary vitamin D [3,8].

Fetal vitamin D status

During pregnancy the renal conversation of the prohormone 25(OH)D to the active form $1,25(OH)_2D$ is enhanced, mediated by an upregulation of CYP27B1 in the kidneys, and not by an upregulation of PTH [9]. The higher status of $1,25(OH)_2D$ rises the intestinal absorption of calcium to a maximum in the last trimester to accommodate the increased requirements for calcium of the fetus [6]. During pregnancy metabolism of $1,25(OH)_2D$ also occurs in the placenta, which expresses the CYP27B1 and CYP24A1 enzymes that catalyze this conversion (Figure 1) [6,7]. Both CYP27B1 and VDR are localized both in the maternal and fetal part of the placenta with highest expression in 1st and 2nd trimester. However, the physiological relevance of placental synthesis of $1,25(OH)_2D$ is not entirely clear, since $1,25(OH)_2D$ does not cross the placenta [6]. Instead the fetus is supplied by vitamin D in the form of 25(OH)D, which is converted to $1,25(OH)_2D$ by CYP27B1 in the kidneys of the fetus [7]. Thus, the maternal and fetal 25(OH)D concentrations are correlated, with a fetal concentration corresponding to approximately 80% of the maternal concentration [3].

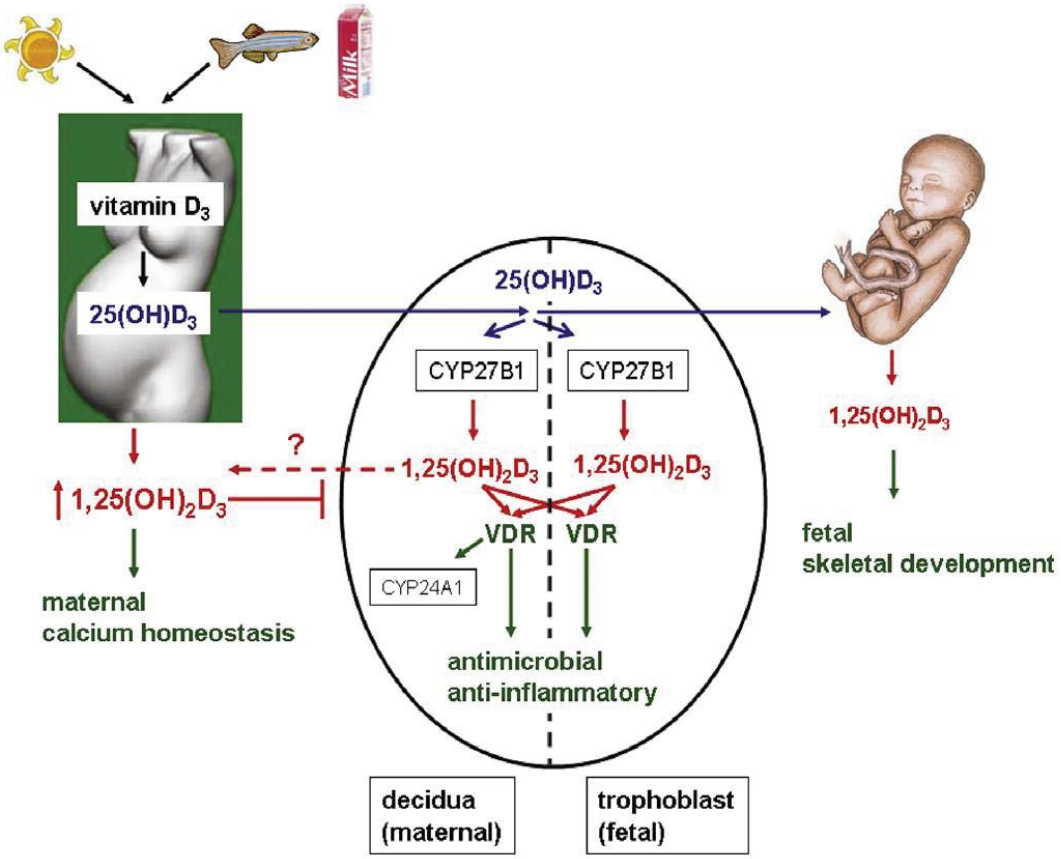

Figure 1. Schematic overview of the vitamin D metabolism and function during pregnancy (adapted from Liu & Hewison [6] with permission).

Maternal vitamin D status during pregnancy

The status of vitamin D in a pregnant woman varies by environment, and is influenced by latitude, cultural habits, food habits, skin color and exposure to UVB radiation [3]. The concentration of $1,25(OH)_2D$ increases during pregnancy, already from 1st trimester and continues up to term [10], while the concentrations of 25(OH)D, $24,25(OH)_2D$, calcium, magnesium and phosphor show little or no change. The levels of 25(OH)D are highly effected by season during pregnancy, with lowest concentration of 25(OH)D during the winter months [11]. Several studies have concluded that a high proportion of pregnant women suffer from vitamin D deficiency [12-17]; however, very few studies have been performed on vitamin D deficiency among pregnant women in the Danish population. A small longitudinal study including 141 Danish pregnant women found that 23.3% had 25(OH)D levels ≤50 nmol/l in 2011 [18], and a case-control study on post-partum depression including 1480 women from the Danish National Birth Cohort (1996-2003) found that 42.3% of the pregnant women suffered from vitamin D deficiency [19,20]. Thus, this indicates that vitamin D deficiency among Danish pregnant women may be of concern.

In general, sufficient vitamin D status is defined as blood concentration of 25(OH)D ≥50 nmol/l [21-24], which is also the guideline for sufficient status during pregnancy [21]. The American Institute of Medicine defines deficiency as 25(OH)D concentrations below 25 nmol/l, and recommends blood concentration of 25(OH)D in the range between 75 and 125 nmol/l [24]. The Danish Health and Medicines Authority has similar guidelines regarding blood concentrations of 25(OH)D [23]. The definition of optimal vitamin D status has been subject to much debate and is still intensively debated worldwide. The definitions applied in this thesis are based on the guidelines from American Institute of Medicine and the Danish Health and Medicines Authority.

Fetal and child bone development

During pregnancy maternal calcium and bone metabolism is changed as early as in gestation week 12 to meet fetal calcium needs for skeletal development [9,25]. The fetus is totally dependent on maternal resources of calcium, phosphorus and magnesium to allow normal mineralization of the skeleton [26]. Despite the high demand for calcium by the fetus, there is limited evidence that low calcium intake during pregnancy affects fetal skeletal development. Rather, it seems the mothers bone mineral content is severely affected by low intake of calcium and other bone minerals during pregnancy [25]. The maximal demand for calcium is in

the third trimester where 80% of the total calcium (of approximately 30 g at term) is accumulated in the fetal skeleton [10,25]. More than 150 mg calcium per kg body weight and 70 mg phosphate per kg body weight are provided every day to the growing fetus. The minerals are transported across the placenta by active transport, and the fetal serum concentrations of calcium and phosphate (both total and ionized fractions) are higher than maternal concentrations [26].

Generation and maintenance of the placental mineral ion transport is regulated by fetal hormones, including vitamin D, PTH, parathyroid hormone-related peptide (PTHrP), calcitonin, prolactin and insulin-like growth factor-1 (IGF-1) [9,25]. During pregnancy there is a twofold rise in maternal intestinal calcium absorption to meet the fetal need for calcium during growth and development. This rise in absorption is mediated by the increase in $1,25(OH)_2D$ [9]. To meet the fetal requirements of calcium the renal excretion of calcium also decreases and the resorption of calcium from the maternal skeleton increases [9]. In general, $1,25(OH)_2D$ is essential for bone growth and bone remodeling by activating bone resorption. Further, it alters the expression of numerous osteoblast genes, the synthesis of matrix proteins that regulate bone mineralization, and directly stimulates osteoblast activation [3].

Second trimester is a critical period for long bone growth in the fetus, by rapid cell division and differentiation via endochondral ossification, where $1,25(OH)_2D$ seems to be essential [27] The development of the long bones are of particular concern, due to fragility fractures in old age mainly occurring in the long bones. Vitamin D deficiency during pregnancy may disrupt the process of fetal bone formation leading to persistent changes in the child's bone structure [28]. Other maternal factors that seems to influence neonatal bone mass include: anthropometry, parity, smoking, age [9,29,30], diabetes mellitus, endocrine imbalance [9,31], maternal alcohol and coffee intake [32-34], and season of birth [9,35]. Two other significant factors are fetal growth restriction in utero [28], and gestational age, because the majority of fetal bone is gained during third trimester [36].

After birth the neonate develops intestinal calcium absorption to maintain normal calcium homeostasis. During the first 24 hours after birth there is a physiologic nadir of both total and ionized calcium in healthy newborns. During the first days of life serum calcium rises most likely as a consequence of stimulation and maturation of the PTH response. [26]. During infancy and childhood the skeleton grows as the body grows, both in length, breadth and in mass [37],

and the rate of skeletal growth is highest during the first year of life. An average infant gains 220 g of BMC in the first year of life, corresponding to a 275% increment [38]. Breast milk has in general a low concentration of vitamin D, and may be an inadequate supply to the growing fetus with sufficient amount of vitamin D [26]. In Denmark a supplement of 10 µg of vitamin D drops per day is recommended from the age of 2 weeks to 2 years to secure optimal vitamin D status [21].

Significance of high peak bone mass

Adult life bone mass is dependent upon the peak bone mass accumulated before the fusion of the long bone epiphyses approximately at age 20 (Figure 2) [27,39]. Approximately 40% of the skeletal mass in females is gained when they are between 12 and 18 years of age [39].

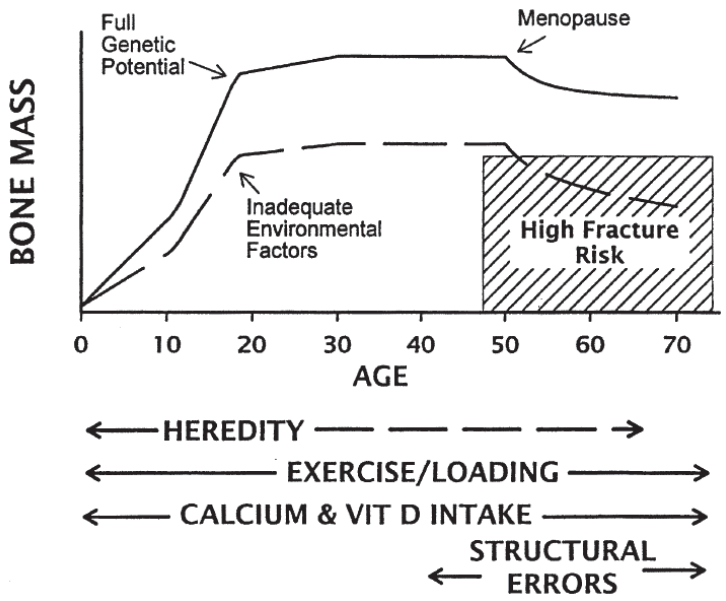

Figure 2: Graphic illustration of the bone mass life-line in individuals, who achieve their full genetic potential for bone mass, compared with those who do not (adapted from [37]).

Through the continuous bone remodeling mature bone tissue is removed from the skeleton and new bone tissue is formed [40,41], however if more bone tissue is removed than what is formed then this imbalance may cause bone fragility and low bone mass. Such imbalance may increase the risk of osteoporosis, a skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, which implies bone fragility and high risk of bone fractures. The disease has become a serious public health issue and a major economic

burden on the health-care system. It is estimated that the number of hip fractures worldwide will rise from 1.7 million in 1990 to 6.3 million in 2050 [42]. Thus, building an optimal peak bone mass during childhood and adolescence can be viewed as a main strategy for reducing the risk of osteoporosis later in life [27,43].

Determinants for improved peak bone mass

Although the main explaining factor with respect to variability of peak bone mass is heritability, lifestyle factors during childhood and adolescence have also been found to influence peak bone mass, including weight-bearing physical activity [39,43]. Physical activity from age 15 years to young adulthood is reported to be the strongest predictor of BMC in adults [44], and physical activity patterns during teenage years is found to account for 10-22% of adult variability in BMC [45]. Smoking, high intake of carbonated soft drinks, alcohol and coffee has also been linked to low bone mass during childhood and adolescence, whereas conflicting results have been reported for the intake of protein, calcium, milk products and vegetarian dietary habits [39,43]. Despite the fact that the intake of dairy products and calcium is high in many developed countries, osteoporosis and fracture rates in those countries are among the highest in the world [39,46]. One other potentially modifiable risk factor that has been hypothesized to be part of the underlying cause of the high incidence rate of osteoporotic fractures in developed countries is vitamin D deficiency [39,42].

Vitamin D deficiency during childhood

The major role of vitamin D in relation to childhood bone mineralization is to ensure normal calcium and phosphate levels in the blood [47]. Children have higher calcium demands than adults, and vitamin D is a crucial factor when assessing the role of calcium intake [48], because vitamin D status interacts with calcium uptake and metabolism, by regulating the absorption of intestinal calcium [48]. Low calcium levels are recognized by the parathyroid gland that releases PTH to activate renal calcium reabsorption to secure serum calcium levels in an adequate range. Meanwhile, the rise in PTH also decreases mineralization of the child's skeletal bones [49]. A recent study including 193 children aged 12-22 months concluded that the blood concentration of 25(OH)D should be more than 60-65 nmol/l to maintain low levels of PTH in young children [50]. This indicates that the recommended 25(OH)D level of ≥50 nmol/l may be inadequate to secure optimal bone mineralization during childhood and adolescence [47].

There is increasing evidence that low vitamin D status is common in children in many developed countries, possibly in consequence of less outdoor activity in combination with more extensive skin covering, sunscreen use and increased obesity [51]. Recent studies from Denmark support that vitamin D deficiency is of concern. The mean concentration of 25/OH)D through the winter was 23.4 nmol/l among 54 Danish girls aged 11-13 years, and 92.6% were insufficient (<50 nmol/l), and 51.9% were deficient (<25 nmol/l) [52]. In a larger population of 834 Danish children at 8-11 years of age blood concentrations of 25(OH)D were measured from September to November, and revealed that 28% were insufficient [53]. However, another study including 340 Danish children aged 4-17 found 5% (4-10y) and 8% (11-17y), respectively, to be insufficient based on measurements from September-October [54]. Also among Danish infants vitamin D deficiency seems to be frequent. A study including 107 pregnant women and their offspring reported insufficient levels in 61% of the newborns [55]. The apparent discrepancy between the studies may reflects the large variability of vitamin D status through the year, rather than actual differences between study populations or age groups.

In extreme cases, prolonged failure or delay in bone mineralization induces osteomalacia in mature bones and rickets in immature bones in children. In general the children are <18 months of age or in the adolescent growth spurt [56,57]. Rickets is characterized by inadequate mineralization of the bone matrix causing limb deformities, beaded ribs, widened wrists and ankles, and leg pain during walking in the growing child [38,56]. In developed countries rickets is a rare disease, that mainly occurs in dark-skinned children from immigrants' families with dark skin color [38]. A study between 1985-2005 supports that rickets is a rare disease in Denmark, but the incidence among ethnic Danish children was unexpectedly high [58]. The average incidence of rickets was 2.9 per 100,000 per year (0-14.9y). Ethnic Danish children were only diagnosed in early childhood, and the incidence declined in the period 1985 to 2005, from 5.0 to 2.0 per 100,000 per year (0-2.9 y). In comparison, the incidence among immigrant children born in Denmark was 60 per 100,000 (0-14.9y) [58,59]. The link between vitamin D deficiency and rickets is well understood; however, the impact of subclinical vitamin D deficiency on bone health in children may also be of substantial importance. Low childhood bone mineralization as a consequence of low vitamin D status lower the acquisition of bone mass and the final peak bone mass, and may in turn contribute to increased bone fracture risk during childhood [60].

Fetal programming of bone health

Fetal programming is an emerging concept that links environmental conditions during embryonic and fetal development with risk of diseases later in life. In 1992 Dr David Barker showed that infants with low birth weight have an increased risk of developing coronary heart disease as adults [61]. This hypothesis for an association between fetal life exposures and risk of diseases later in life was in 1995 named the "Barker Hypothesis" by the British Medical Journal [62], and it is now widely recognized. Multiple data from epidemiological observations as well as clinical and experimental studies support the concept of fetal programming.

A hypothesis for fetal programming of bone health was established in 2001 following the results of an observational study that showed a close relation between childhood growth and hip fractures in old age in a Finish birth cohort from 1924-33 [27,63]. Since then, several different lines of evidence have supported the hypothesis that the risk of osteoporosis in adulthood may be modified by environmental influences during fetal life. Several studies have addressed the hypothesis of fetal programming of bone health. This has mainly been observational cohort studies where bone mineral measurements in adults are related to detailed birth and/or childhood growth records, studies that relate childhood growth to later risk of bone fractures, and studies where nutritional and lifestyle style factors during pregnancy are related to bone mass in their newborn offspring [36]. However, few studies have investigated whether maternal diet and especially maternal vitamin D status during pregnancy may impact offspring bone health in short as well as in the long term.

In table 1 and 2 are summarized the literature in the field regarding the potential aspects of fetal programming of bone health in relation to maternal nutrition and vitamin D status during pregnancy. Overall, the literature in the field is limited [64]. There is some evidence for higher bone mass in the offspring if the mother during pregnancy ingested a diet low in total fat, but high in calcium and magnesium. Also the status of folate and vitamin D seems to be of importance. However, many of the studies have been small and only one has followed the offspring beyond the first ten years of life. The studies have primarily analyzed the associations between single food items and nutrients, and not with dietary patterns. Thus, their results may be prone to confounding by other underlying foods or nutrients that may potentiate or attenuate the effect of others.

Table 1: Studies in the field investigating the association between vitamin D status during pregnancy and measures of offspring bone health.

Study	Population	Subjects	Exposure	Measurement	Outcome	Conclusion
Javaid et al.[65] 2006	Princess Anne Cohort Ctudy	160 offspring at 9 years of age	25(OH)D in gestation week X	DXA (whole body, lumbar spine)	BMC, BMD	Positive association
Ioannou et al.[66] 2012	Southampton Women's Survey	357/203 neonates	25(OH)D in gestation week 34	Three-dimensional ultrasound in gestation week 19-24 (femur)	Femur volume	Positive correlation
				DXA at 0-2 weeks of age (whole body)	BMC, BMD	No association
Sayers & Tobias [67] 2009	Avon Longitudinal Study of Parents and Children (ALSPAC)	6995 children at 9.9 years of age	Estimated UVB radiation in third trimester	DXA (total body less head)	BMC, BMD, BA	Positive association
Lawlor et al.[68] 2013	ALSPAC	3960 offspring at 9-10 years of age	25(OH)D from the latest blood sample in any stage of the pregnancy	DXA (total body less head, spine)	BMC	No association
Zhu et al.[69] 2014	Australian Raine Study	341 offspring at 20 years of age	25(OH)D in gestation week 18	DXA (total body)	Peak bone mass	Significantly decreased peak bone mass when 25(OH)D was < 50 nmol/l

Table 2: Studies in the field investigating the association between maternal diet during pregnancy and measures of offspring bone health.

Study	Population	Subjects	Exposure	Measurement	Outcome	Conclusion
Jones et al.[70] 2000	Cohort from Southern Tasmania	173 offspring at 8 years	Maternal diet in third trimester	DXA (lumbar spine)	BMD	Total fat ↓ Phosphorus ↑
Tobias et al.[71] 2005	ALSPAC, UK	4451 children at 9 years	Maternal diet in late pregnancy	DXA (total body and spine)	BMC, BMD	Magnesium ↑ Dietary folate ↑
Ganpule et al.[72] 2006	Indian cohort	698 children aged 6 years	Maternal diet measured in 18 and 28 gestation week	DXA (total body and spine)	BMD BMC, BMD	Folate ↑ Calcium-rich foods ↑
Chang et al.[73] 2003	African birth cohort	350 African teenage mothers	Maternal diet, 20- 34 gestation week	Ultrasound (femur)	Fetal femur length	Dairy ↑
Yin et al.[74] 2010	Tasmanian birth cohort	216 adolescents at age 16	Diet in third trimester	DXA(femoral neck, lumbar spine, total body)	BMD	Total fat ↓ Magnesium ↑ Milk ↑
Cole et al.[75] 2009	Princess Anne cohort study, UK	198 offspring at 9 years of age	Diet in first trimester	DXA (whole body, lumbar spine)	BMD	Prudent diet ↑ (characterized by high intakes of fruit, vegetables, dark bread, rice, pasta, yoghurt, breakfast cereals)
Heppe et al.[76] 2013	Generation R study, the Netherlands	2819 offspring at 6 years of age	Diet in first trimester, venous blood concentrations of homocysteine, B12 and folate (around gestation week 13)	DXA (whole body)	BMC	Phosphorus ↑ Homocysteine ↑ Protein ↑ Vitamin B12 ↑

↓ = Negative association

↑ = Positive association

Pediatric bone fractures

The epidemiology of pediatric bone fractures has received very little attention, while bone loss and fracture risk in old age are much more studied [37], though fracture incidence during childhood is similar to that in the elderly [77]. Childhood bone health is normally measured using dual energy x-ray absorptiometry (DXA), a technique that estimates BMC and bone area (BA) from which BMD is calculated by dividing BMC by BA. One disadvantage by using DXA is that the measurements can be affected by the subjects' size, and there is no consensus on how to correct the results for size. Therefore, the use of uncorrected measurements, may introduce bias in analyses of causal relations in epidemiologic research studies [77,78].

There seems to be a close correlation between DXA-derived measures of BMD and bone fractures in children. This indicates, that propensity of bone fractures can be used as a measure for childhood bone health. A meta-analysis of ten case-control studies showed an association between BMD and bone fractures in children [78], and review of the existing literature have indicated consistent and convincing evidence for an association between BMD and forearm fractures in children, even if falls and injuries are common and can be attributed to normal child development [79]. The risk of fractured bones varies according to many different factors and does not only rely on BMD. Such factors may be age, sex, socioeconomic status, level of physical activity, season, obesity and risky behavior [80].

Fracture patterns and fall characteristics in children vary by age. The pediatric skeleton has fundamental physiological differences in weight distribution and body structure compared with adults. In infants and toddlers the weight is proportioned towards the head, which makes the head and neck more vulnerable to injury from falls. When the child gets older it more likely brace falls with arms or legs, leading to more extremity and vertebral fractures [80,81]. Thus, small children have higher rates of fractures in skull and clavicle, whereas adolescents have higher rates of fractures in the hand and lower leg [82]. Overall, forearm fractures are the most common type of long bone fractures in children and adolescents, representing 25-30% of all fractures in these age groups [79,83]. The main causes for these fractures are falls with mild and minimal force injury; such as falls from furniture at 0-4 years of age, from playground equipment at 5-9 years of age, and during organized sports activities at the age of 10-17 years [84]. Approximately 10,000 annual pediatric fractures are recorded in the Danish National Patient Register (DNPR) [85].

The patterns of fracture rates differ between boys and girls, reflecting the differences in growth and behavior between the sexes. In general boys have a higher incidence of fractures compared with girls, which may reflect differences in risky behavior between the sexes. The peak for incidence of fractures is during the pubertal growth spurt, where the bone mineral density is decreased because of bone expansion and insufficient bone mineralization [86]. Among girls the peak incidence for fractures occurs at 11 years while it occurs at 14 years of age in boys (Figure 3) [83,86,87].

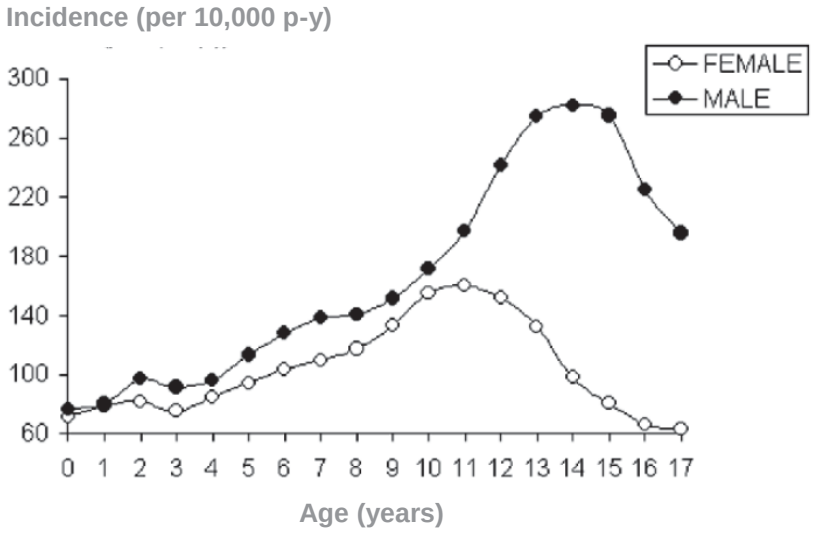

Figure 3: Age and sex-specific incidences of fractures at any site registered in the General Practice Research Database (1988-1998) including children aged 0-17 years from the general UK population (adapted from [83]).

Approximately half of all children will experience at least one bone fracture, and the overall incidence rate of pediatric bone fractures seems to be increasing, both in developing and developed countries; however, the reason for this is not known [80,86]. In Sweden the incidence of pediatric fractures was found to double from 1950 to 1979 [88], and to have increased by 13% from 1998 to 2007 [86]. In a US study from Minnesota the incidence of distal forearm fractures increased by 32% in males and 56% in girls from 1969 to 2001. However, whether this increase in fractures is due to registration error or changes in registration guidelines in pediatric records, decreased vitamin D status during childhood, changes in physical activity patterns or increased obesity among children and adolescents is not clear.